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Morphological, Molecular and Biochemical Comparative Studies of Two Novel Fungal Honey Isolates

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAE has suggested the point and authors MAE, ALK and ZHK have written the protocol. Authors MAE, ALK, HAEA and EKAEH have designed the study and managed the literature searches at the beginning. Author MAE and TK have wrote the first draft of manuscript and author EKAEH has carried out the experiments. All other authors have supervised the thesis and experimental work and managed the literature searches needed during writing the script, discussing the results and publishing. All authors read and approved the final manuscript.

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ABSTRACT

Aims and Study Design: The stress honey medium expected to have spores with unique feature. Within this context, two honey isolates were identified, characterized, and evaluated as secondary metabolite producers.
Place and Duration of Study: The study was undertaken in the National research center, 33 Al Behous, Dokki, Giza, Egypt. The duration of study was during the period of January 2012 to

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February 2013.
Methodology: Two strains were isolated from mountain honey, Yemen. They were identified based on morphological characterization and 18S rRNA sequence analysis. Validation of novelty was supported further by their protein profile and randomly amplified polymorphic DNA PCR patterns (RAPD). Growth parameters were studied to determine optimum growth factors. In addition, they were undergone to enzymatic, antibiotic, antioxidant, ochratoxins and aflatoxins testes.
Results: Two novel honey isolates were identified and designed as *Aspergillus niger* EM77 and *Aspergillus awamori* EM66. Intracellular protein profiles showed clear difference among the honey isolates and their references. The RAPD referred to 51% coincidence between the two isolates and showed different RAPD patterns between the references and honey isolates. Different parameters which affected isolates growth such as pH, temp, carbon and nitrogen sources were studied. Effect of NaCl concentrations referred to the halo-tolerant feature of both isolates. Both strains showed strong antimicrobial activity against *Candida albicans*, *C. tropicalis* and *Pseudomonas* species. Reducing power assay reported 82% and 83% antioxidant activity of *Aspergillus niger* EM77 and *A. awamori* EM66, respectively. Aflatoxins and ocratoxins tests revealed that both isolates had negative results. In addition, two fungal honey isolates showed relatively similar enzymatic activities.
Conclusion: This research continues in highlights the honey as a new reservoir of unique isolates. In addition, it was suggested that the honey osmophilic stress carried spores have novel properties.

Keywords: Aspergillus awamori; Aspergillus niger; polymerase chain reaction; secondary metabolites; enzymes.

1. INTRODUCTION

Previously, honey reports focused in the antimicrobial, antioxidant and anticancer agents of honey, but until now there is insufficient information concerning honey isolates characterization and their bioactive properties [1,2]. Knowledge concerning the moisture and temperature conditions influencing microorganisms' growth in honey has long been used to control the spoilage of honey. Honey microorganisms usually come from pollen, the digestive tracts of honeybees, dirt, dust, air and flowers. However, it is important to note that honey frequently contains dormant endospores. Most of these organisms are said to be in inactive forms as they can hardly survive in honey because of its several properties including hygroscopicity, hyperosmolarity, acidity, peroxide content, antibiotic activities etc. Most of the honeybee isolates were identified as *Bacillus* spp. and showed very strong activity against honeybee pathogens [3]. Esawy et al. [4] reported in six *Bacillus subtilis* isolated from different honey sources. The six isolates were highly potential producer of antibiotic and antioxidant activities. In addition, it was mentioned that aerobic spore-forming *Bacillus* spp. was the most frequently encountered microbe on the external surface, crop and intestine of honeybees [5]. The spores of *Clostridium botulinum* are found in a fraction of the honey samples tested normally at low levels. No vegetative forms of disease-causing bacterial species have been found in honey. The fungi strains isolated from different honey sources were studied by many researchers. Felšöciová, et al. [6] carried out characterization studies for some honey samples from Poland on the basis of their microbiological (fungi and yeasts) analysis. The isolated fungi were *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Fusarium* spp., *Mycelia sterilia*, *Rhizopus* spp. and *Penicillium* spp. The status of the microorganisms found in honey in the spore form called dormant [6]. The knowledge regarding the diversity and functions of honey microorganism's community remains limited. However, the need for additional microbiological data on honey will increase as new technologies for, and uses of honey develop [3]. The high osmolality, acidity and antimicrobial properties of honey, recommended their microorganisms to have new feature and properties [4].

The *Aspergillus* genus belongs to a filamentous fungal group characterized by wide dispersion in the environment. Some species are associated with diseases, while others are of economical importance due to biotechnological applications. Until this moment, there are rare reports in Aspergilli. as honey isolates [7]. Filamentous fungi such as *Aspergillus niger* are well known for their exceptionally high capacity for secretion of proteins, organic acids, and secondary metabolites and they are therefore used in

biotechnology as versatile microbial production platforms [8]. Black-spored aspergilli are difficult to classify and the taxonomy of this section is still unclear [9]. A diagnostic phenotypic procedure based on biochemical traits on agar media along with some molecular approaches has been recently reviewed [10]. The 18S rRNA gene is a molecular marker helps in introducing a large number of sequences available in the data banks [11]. SDS PAGE protein pattern analysis is an easily method to distinguish between *Aspergillus* spp. This technique can be successfully implemented with proper assessing techniques to evaluate the degree of similarity among species of the same genus or of closely related genera [12]. The RAPD method has several unique advantages: it is extremely fast (yielding results within 48 h), it has potential to discriminate between closely related strains (provided multiple primers are used), it works well with crude DNA lysates hence eliminating the cost of extensive purification, and it has been used with great success for several types of fungal species [13]. Moreover, it has a role in evaluation the novelty of strains [14].

Until now, there is scarce information concerning enzymes from honey microorganisms [1]. Last few years, researchers have paid attention to the enzymatic products of the honey isolates. Recently, it was reported in levansucrase from six novel honeys *Bacillus subtilis* isolates capable of producing antiviral levans [1]. On the other hand, a new isolate was identified as *Aspergillus* sp. M1; was isolated from honeycomb and characterized as a good invertase producer [15]. Similarly, the dextranase production from *Bacillus subtilis* KNRC honey isolates was mentioned by Esawy et al. [2].

Honey has natural antioxidant properties that could destroy biologically destructive chemical agents, which have been linked to many diseases such as cancer, also the antimicrobial activity of honey and its role in wounds is well known. The antioxidant and antimicrobial studies of honey isolates activity were still uncommon. Previously, bacillomycin F produced by a bacterial honey isolate was purified and showed antimicrobial activity against *Byssachlamys fulva* [5,4]. On the other hand, Esawy et al. [2] showed antioxidant and antimicrobial properties of six *Bacillus subtilis* honey isolates.

Moreover, the species of filamentous fungi produces several secondary metabolites; one of the most important was ochratoxin A., as an abundant food-contaminating mycotoxin. Mycotoxins are secondary fungal metabolites having toxic response with well-known health effects including; carcinogenesis, and immune-suppression [16]. Nevertheless, studies have shown that less that 10% of the *A. niger* strains were tested positive for ochratoxin A, under conditions that were favorable [17].

In the present study, two fungal strains were isolated from mountain honey, the morphological and molecular identification by18SRNA were achieved. The protein pattern and the fingerprint were done to distinguish between the two isolates. In addition, the effect of different temperature, pH, carbon and nitrogen sources, and NaCl concentrations were studied. Finally, the antioxidant, antimicrobial and some enzymatic activities of the fungal strains were investigated. The results pointed to the isolates novelty.

2. MATERIALS AND METHODS

2.1 Sample Collection

Two isolates were isolated from Yamane honeybee collecting nectar from mountain flower. Honey samples are fresh non-treated ripe honey (directly collected in beehives) in sterile vials.

2.2 Isolation of Fungal Strains from Honey Samples

Honey samples one hundred micro liters was spread on Czapek Dox agar plates (g/L): Sucrose, 20.0; Na_2NO_3, 2.0; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01 and Agar, 20.0; pH 6. The plates were incubated at 30℃ for 72h. The individual colonies were transferred separately to the same medium and the growth was observed for 72h. The selected fungal isolates were maintained onto Czapek Dox agar plates and preserved at 4℃. The purity of the fungal isolates was assessed by colony morphology and microscopy [18].

2.3 Identification of the Two Isolates

For morphological identification, filamentous fungi were initially cultured on PDA medium (Merck) (g/L): 200 potatoes (sliced washed unpeeled), 20 dextrose, 20 agar. The colonies were observed with an optical microscope for preliminary identification. This was done by morphotypic analysis of the colony, especially

color and appearance, using the method of Pitt & Hocking [19]. Initial identification of the genera *Aspergillus* was made with microscopic slide examination of spores and mycelium.

2.4 Molecular Analysis

Culture and collection of the mycelium was carried out as reported by Zhao et al. [20]. For sequence analysis DNA was extracted from the tow isolates using a fungal sequences DNA isolation kit (RKT13: Chromous Biotech, India). The 18S rDNA sequences were amplified by (polymerase chain reaction PCR) with primer pairs 413 bp. (GTGGGGGTAGGATGAGATGATG) and BA1r (TGAGTGCTGGCGGAAACAAA). QIA quick PCR purification kit (Qiagen, Germany) was used to purify the amplified products of BA2f/BA1r specific primer of 20 fungal isolates.

A GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT) thermo-cycler device was used with the following program: 94℃ for 5min, as initial denaturation cycle and 35 cycles consisting of denaturation at 94℃ for 30 sec, annealing of primer at 60℃ for 30 sec and extension for 1 min at 72℃ and finally addition of 3` terminal at 72℃ for 10 min.

2.5 PCR Amplification

PCR amplification was performed according to Thompson et al. [21] in a total reaction volume of 25 µl. The nucleotide sequences were edited and carried out using CLUSTAL/W (1.82) http://www2.ebi.ac.uk/clustalw [21]. Bootstrap neighbor-joining tree was generated using MEGA version 3.1 from CLUSTAL/W alignments [22]. Comparisons with sequences in the GeneBank database were achieved in BLASTN searches at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlMnih.gov).

2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis for Separation of Denatured Proteins

The extracts of fungi were analyzed by making use of SDS-PAGE method with 12% separating gel and stacking gel 5% in a discontinuous buffer system according to the method of Laemmli [23]. Thirty µl of the protein extract were mixed with 5 µl of sample buffer, then heated in a boiling water bath for 3 minutes for protein denaturation. Electrophoresis was carried out using Hoefer Vertical Slab gel unit, Model SE-400. The prepared samples were loaded on the gel at constant Voltage of 150V and 15 mA, until the bromophenol blue dye reached the bottom of the gel. The duration of the separation was about 5-6 hours. The gel was directly placed in the Coomassie brilliant blue staining solution overnight. The gels were destained several times for twelve hours in the destaining solution. After destaining, the gel was photographed, dried and kept for comparison.

2.7 Subunit Molecular Weight Estimation by SDS-PAGE

The labimage program was used to determine the apparent (subunit) molecular weight of proteins dissolved or extracted in the presence of SDS. Electrophoretic mobility's were calculated relative to the mobility of the bromophenol blue marker band in 12% and 5% polyacrylamide slab gel. The following proteins were used as molecular weight standards for the samples: (232.0 KDa), (218.0 KDa), (210.0 KDa), (208.0KDa), (180.0KDa), (175 KDa), (133 KDa), (130 KDa) , (122 KDa), (115 KDa), (110 KDa), (96 KDa), (92 KDa), (87 KDa), (81 KDa), (77 KDa), (76 KDa), (74 KDa), (72 KDa), (71 KDa), (67 KDa), (65 KDa), (60 KDa), (59 KDa), (47 KDa), (45 KDa), (41KDa), (39 KDa), (38 KDa), (37 KDa), (36 KDa), (35 KDa), (33 KDa), (19 KDa), (17 KDa), (8 KDa), (7 KDa), (5 KDa), (2 KDa).

2.8 Differentiation of Two Isolates by Finger Printing (RAPD-PCR Conditions and Analysis)

Twenty-three short- mer arbitrary primers were used for RAPD analysis. Sequences of all primers are:

G-17 5'-ACGACCGACA-3**,** H-18 5'-GAATCGGCCA-3', H-01 5'- GGTCGGAGAA-3', B-10 5'- CTGCTGGGAC-3', Q-15 5'-GGGTAACGTG-3', Z-02 5'-CCTACGGGGA-3', H-03 5'-AGACGTCCAC-3', M-18 5'-CACCATCCGT-3', G-04, 5'AGCGTGTCTG-3', Z-08 5'-GGGTGGGTAA-3', Z-02 5'-CCTACGGGGA-3', H-01 5'- GGTCGGAGAA-3', M-18 5'- CACCATCCGT-3', Q-15 5'-GGGTAACGTG-3', H-18 5'-GAATCGGCCA-3', Q-07 5'-CCCCGATGGT-3', Q-09 5'-TCCCACGCAA-3', Q-08 5'-CCTCCAGTGT-3', Z-07 5'-CCAGGAGGAC-3', M-05 5'-GGGAACGTGT-3', G-11 5'-TGCCCGTCGT-3',

O-18 5'-TGCCCGTCGT-3', H-08 5'-GAAACACCCC-3'

Aspergillus niger obtained from Taxonomy Department, Ain Shams University, Cairo, Egypt and *Aspergillus awamori* is commercial isolate from Japan. The both are soil isolates. They were used through this study as reference and marked as C and C^w, respectively.

For RAPD analysis, PCR amplification was carried out in a 25 µl final volume containing; 2.5 µl 10 x PCR buffer, 1 µl 10 mM Mg Cl_2, 1 µl 2.5 mM dNTPs (for each), 1 µl (50 pmol) of primer, 1 µl (approximately 50 ng) of fungal template DNA, 0.3 µl (5 units μl^{-1}) *Taq* DNA polymerase (Promega, Germany) and 18.2 µl sterile distilled H_2O. PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for one cycle at 94°C for 5 min followed by 35 cycles each with 30 s at 94°C for denaturation, 1 min at 30°C for annealing and 2 min at 72°C for extension. Reaction mixture was then conducted at 72°C for 10 min for final extension and hold at 4°C.

Five µl of the PCR amplification products were mixed with 2 µl of 6X gel loading dye and loaded onto1.5% agarose/0.5X TBE gels. One kbp DNA ladder (New England Biolabs) with size marker, ranged from 100 to 1000 bp was used as molecular size standard. Electrophoresis was performed at 100 Volt with 0.5X TBE as running buffer. The gel was stained in 0.5 µg/ml (w/v) ethidium bromide solution and distained in deionized water for 10 min [24] and visualized on an ultraviolet dual intensity trans illuminator (WVP, USA), and photographed using gel documentation system (WVP, USA).

2.9 Data Handling and Cluster Analysis

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. Presence and absence of RAPD bands produced from the use of four primers were scored visually from the resulting photographs.

If a product was present in fungal genotype, it was designated '1', and if absent it was designated '0' after excluding un-reproducible bands. Pair-wise comparison of genotypes, based on the presence or absence of unique and shared polymorphic products, was used to generate similarity coefficients, which were used to construct a dendrogram by UPGMA (Unweight Pair-Group Method with Arithmetical Averages) using NTSYS-PC software [25].

2.10 Fermentation Conditions

The Czapek Dox (CMF) liquid medium (mentioned above) was used for enzyme production under submerged fermentation (CMF). Cultivation was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium After inoculation (10^6 spores/ml), the flasks were incubated at 30°C for three days in an incubator shaker at 150 rpm At the end of fermentation period, the supernatant was harvested by filtration and was used as crude enzyme extract. The mycelia mass was collected and its dry weight was determined.

2.11 Effect of Different Growth Parameters

2.11.1 Temperature

The cultivation medium Czapek Dox (CMF) was incubated at different temperatures (25, 30, 35, 40 & 45°C) for 72h to study their effect on the fungal isolates growth.

2.11.2 pH values

In this experiment, the pH of culture medium was adjusted to pH (2.0-9.0) by using 0.1 N HCl or 0.1N NaOH. The cultivation medium (CMF) was incubated at 30°C for 72h.

2.11.3 Carbon sources

In order to measure the effects of different carbon sources on fungal isolates growth, the sucrose in CMF was replaced by 2% (w/v) of different carbon sources (Fructose, Galactose, Mannose, Lactose, Casein, Glucose, Cellulose and pectin).

2.11.4 Nitrogen sources

Addition effect of equal-molar amounts of different inorganic nitrogen ($NaNO_3$, NH_4NO_3, $(NH_4)_3PO_4$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, NH_4Cl, KNO_3) and organic nitrogen (Yeast extract, Peptone) sources on fungal isolates growth was also studied.

2.11.5 NaCl concentrations

In this experiment, the salinity effect on the growth of fungal isolates was carried out by growing the organisms in CMF medium

contained different final concentrations of NaCl (2, 4, 6, 8, 10, 12 & 14%) at the same previous conditions.

2.12 Antioxidant Property (Radical Scavenging Activity)

The assay of di-phenyl-picryl-hydrazyl (DPPH) was carried out according to Bhandari and Kawabata [26]. Aliquots (2 ml) of the fungus isolates spore suspension were added to 50 ml of CMF medium, and incubated at 30℃ for one day. The medium were centrifuged; 1 ml of the filtrate was lyophilized, and then re-dissolved in 1 ml of methanol. Fifty-µl volumes of methanolic extract were added to 5 ml of 0.004% methanol solution of DPPH. After 50 min incubation period at dark, the absorbance was read against a blank at 517 nM Free radicals DPPH inhibition in percent was calculated as follows:

$$I\ (\%) = (A\ blank - A\ sample/A\ blank) \times 100$$

Where: A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test sample.

2.13 Antimicrobial Properties

The antimicrobial tests were carried out by the disc-diffusion method [27]. Each culture of both isolates was cultivated into 50 ml of nutrient broth media, incubated shaking at 30℃ for 3 days. The media were centrifuged, the filtrates were extracted by 150 ml ethyl acetate several times, then dried under reduced pressure at 40℃ and 50 µg of culture extract were dissolved in one ml ethyl acetate. One hundred µl of the culture extract were used to saturate filter paper discs (6 mm diameter) and placed gently on the surface of nutrient agar medium previously inoculated with one ml of cell suspension of clinical tested strains. Negative controls were prepared using the ethyl acetate alone. The plates were incubated at 30℃ for 72 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

2.14 Enzymatic Assays

The amylase, invertase, levansucrase, dextranase, inulinase, pectinase, and exochitinase activities were determined**.** **Amylase** activity was done according to the method of Bergmann et al. [28]. One unit of enzyme activity (U) was defined as the amount of the enzyme liberating one µmole of reducing sugars as glucose/min. **Levansucrase** assays were performed according to the method of Yanase et al. [29] with some modifications [30]. One unit of enzyme activity was defined as the amount of enzyme that produced decreasing sugars equivalent to one mol glucose/min**.** **Invertase** activity was measured by the release of reducing sugars according to Miller 1959 [31]. One unit of enzyme activity (U) was defined as amount of enzyme that releases one µmol of glucose per min under the assay conditions**.** **Dextranase** activity was measured using the Somogyi-Nelson method [32] and dextran of molecular weight 250 KDa was used as substrate. One unit (U) of enzyme is defined as the amount of enzyme, which liberates one µmol of glucose equivalent in one min. **Inulinase** activity was measured using the Somogyi - Nelson method [32]. One inulinase unit was defined as the amount of enzyme liberating one micromole of fructose equivalent per minute under the tested conditions. The **pectinase** activity was determined using by the Somogyi-Nelson method [32]. One unit (U) of pectinase activity was defined as the amount of enzyme producing one µmol galacturonic acid per min**.** **Exochitinase** activity was determined according to the method of Matsumoto et al. [33] using the chromogenic substrate p- nitro phenyl-β-D-N-acetyl glucose aminide (PNP-β-GlcNAc) as a substrate One unit of the enzyme activity was defined as the amount of enzyme releasing 1µmol of P-nitrophenol per minute under the specified assay conditions.

2.15 Determination of Aflatoxins by HPLC

Derivatization: The derivatives of tested samples and standards were done as follow:

Two-hundred µl hexane were added to the clean up dry film of standard and tested samples followed by 50µl Trifluoroacetic acid (TFA) and mixed by vortex vigorously for 30s. The mixture was let to stand for 5 min. To the mixture, 450 ml water-acetonitrile (9+1, v/v) was pipetted into the mixture and mixed well by vortex for 30 seconds, and the mixture was left to stand for 10 minutes to form two separate layers. The lower aqueous layer was used for HPLC analysis (AOAC 2000).

An isocratic system with water: methanol: acetonitrile 240:120:40 [34]. The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µL for both standard solutions and sample extracts. The fluorescence detector was operated at

wavelength of 360 nm for excision and 440 nm for emission.

Quantitation: The mixed solutions of standard as well as sample extract after derivatization were filtered through a 0.22 mm membrane filter and loaded (20 mL) into a 20-µL injection loop. The elution order of the four aflatoxins was G2, B2, G2a (G1 derivative), B2a (B1 derivative). AFs contents in samples were calculated from chromatographic peak areas using the standard curve.

2.16 Determination of Ochratoxin by HPLC

The fore-mentioned columns elutes were dissolved in 500 µl mobile phase consisted of acetonitrile: water: acetic acid (99:99:2) and filtered through 0.45 µm micro-filter into 5ml screw-capped vial for subsequent HPLC analyses. High performance liquid chromatography (HPLC) was used to ochratoxins A determination. The system equipped with (Waters 600) delivery system HPLC column; a reverse phase analytical column packed with C_{18} material (Spheris orb 5 µm ODS2, 15cm×4.6nm). The detection was performed using the fluorescence detector, which was operated at an excitation wavelength of 330 nm and an emission wavelength of 460 nm .The separation was performed at ambient temperature at a flow rate of 1.0 ml/ min. Data were integrated and recorded using a Millennium Chromatography. Manger Software 2010 (Waters, Milford MA01757). Quantitation: Calculated from chromatographic peak areas using a standard curve.

3. RESULTS AND DISCUSSION

Extreme environments have long been considered as the best medium for unique microorganisms. Thus, the researchers paid attention to honey isolates characteristics and their bioactive products [1,35,15].

The present study aimed to reply the question of whether the honey dormant spores have properties or features recommended them to be considered as unique strains. Within this context, two fungi were isolated from Yamane mountain honey on mature stage. They were identified based on their morphological examination and the results were confirmed by using 18SrRNA gene sequences. The microscopic examination of the two strains revealed that the isolate (A) and isolate (E) fungi were belonging to the *Aspergillus niger* and *Aspergillus awamori* respectively (Table 1). It is being increasingly recognized that comparative sequence based methods used in conjunction with traditional phenotype based methods could offer better resolution of *Aspergillus* species [36]. Accordingly, 18S rRNA gene sequence was used. The aligned sequence of this amplified 566 bp region of the 18S rRNA segment from isolate A and 640 bp 18S rDNA segment from isolate E was submitted to Genbank. After homology searching against the Genbank, the sequences of isolate A was found to share 96% similarity with those of *Aspergillus niger* and isolate E was found to shear 95% similarity with those of *Aspergillus awamori*. They were designed as *Aspergillus niger* EM77 (KF774181) and *Aspergillus awamori* EM66 (KF774180). A phylogenetic relationship was established through the alignment and cladistic analysis of homologous nucleotide sequences among these fungal for both isolates (Figs.1 a,b). The similarity percent less than 97% pointed to the novelty of both isolates. The 18S rRNA gene as a molecular marker offers the advantage of a large number of sequences available in the data banks [10]. The following part aimed to compare between the two stress honey isolates and their references from normal soil medium (Fig. 2). Data analyzed by the gel document system (GD), indicated that the total number of protein bands of honey isolates and its references ranged from 14-18 band (Table 2). In general, the results pointed to unique characteristic profile of each honey isolate. In *Aspergillus niger* EM77 and its reference the pattern of bands suggested the presence of two common bands in the stressed and normal isolates. The first and second bands were at 92KDa and 38KDa, respectively. The honey isolate showed ten new bands with different MW compared with its reference. In *Aspirgillus awamori* EM 66 and its reference the pattern of bands suggested the presence of seven common bands. They were at 122, 92, 65, 55, 33, 8, 2 KDa. It revealed new eleven bands with different MW as it compared with its reference. The new bands could be due to the response of these isolates to honey osmophilic stress. Also, it was an indication to the novelty of these isolates.

It was found that although RAPD-PCR analysis could be applied as a simple, rapid, and very effective method for differentiating *Aspergillus* strains, selecting the best primers is a critical step to reach the desirable potential discrimination [37]. RAPD analysis is also used

to confirm the strain novelty [38]. Accordingly, the random amplified polymorphic DNA (RAPD) was implemented by using 10 primers, a total of different reproducible RAPD markers were generated from the isolate genomes. Record of presence or absence of molecular weights corresponded to RAPD primers, was evaluated. In addition, 10 primers gave representative profiles for the fungal isolate clusters (Fig. 3a). In RAPD-PCR analysis by using 10 primers, different reactions were generated from both fungal isolates. It was concluded that distinct RAPD fingerprints among the different species were obtained when suitable primers were used [39]. According to these results, the RAPD technique indicated 51% coincidence between fungi A and fungi E, indicating a relative similarity between these two isolates. Our results were in agreement with Ferracin et al. [40]. Randomly amplified polymorphic DNA (RAPD) patterns of whole-cell lysates from *Aspergillus niger* EM77 including one reference (*A. niger,* soil isolate*)* was done (Fig. 3b). The result revealed polymorphism in 51% between the two isolates. In addition, RAPD patterns of whole-cell lysates from *Aspergillus awamori* EM66 by using *Aspergillus awamori* soil isolate as reference was implemented (Fig. 3c). The result revealed polymorphism in 58% of the two analyzed *Aspergillus awamori*. This result indicated clearly the novelty of the two isolates and it could be attributed to the presence of these isolates under stress, which affects the molecular structure of its dormant spores to adapt its harsh environment. On contrary, [41] showed RAPD patterns of whole-cell lysates from five *Aspergillus niger* isolates, including one reference strain, two isolated from deep freeze, and two environmental strains from soil and plant infections. Comparison of deep freeze isolates showed identical RAPD patterns in some of the reference and environmental isolates.

The following part aimed to evaluate different parameters, which affected the isolates growth and summarized it in Table (3). The results indicated that the optimum pH and temperature for the maximum growth were the same for both fungal isolates (pH7 and 30℃). Below and above the previous values the fungi growth decreased gradually. In addition, the results indicated the isolates ability to grow in wide pH range (2-9).

On the other hand, both isolates have the ability to grow in all the used carbon and nitrogen sources. In addition, the results cleared that most favorable carbon source for both isolates growth was mannose, followed by pectin and cellulose Moreover, the best nitrogen source for both strains was casein, followed by NH_4Cl. These results confirmed the similarity between the two isolates. Similar result was obtained by Kosalková et al. [42], since *Aspergillus awamori* achieved increase (40 to 80-fold) in mass production with casein or casein phosphopeptides (CPPs). On the other side, it was reported that maltose supported *Aspergillus niger* growth substantially more than either sucrose, glucose or fructose [43]. For determination the effect of NaCl on the two isolates growth, they were cultivated in the presence of different NaCl concentrations (2-14%). The results showed that both of them were characterized by the halo-tolerant feature, since the *Aspergillus* EM77 (KF774181) and *Aspergillus awamori* EM66 (KF774180) could grow in the presence of 10 and 12% NaCl respectively. Above this concentration, none of the tested fungi could grow (Table 3). Many authors talk about the halotolerant property of *Aspergillus* sp. [44].

Filamentous fungi have been identifying as promising hosts for the production of recombinant proteins due to their desirable growth characteristics. They can produce and secrete exceptionally large amounts of protein [45]. Synthesis of enzymes depends on the type of nutrients available to the organism and besides an adequate carbon source; other nutrients could be equally important to the composition of the medium [46,47]. Within this context, some enzymatic activities studies were achieved. The results referred to the ability of both isolates to produce different amounts of important enzymes (Table 4). The most pronounced activity was noticed with pectinases, levansucrase and chitinases. This result explained clearly the high mass production for the tow fungi in the presence of pectin as substrat. It was mentioned by Carpita and Gibeaut [48] that *A. niger* produced pectinase as major enzyme to degrade pectin though there are many other enzymes also secreted but the prominent one is pectinase [49]. On the other side, many authors reported in *Aspergillus awamori* as a good pectinase producers [50]. This result confirmed the potentially of both isolates in producing important enzymes and referring to its unique characteristic.

Fungi are known to produce a vast array of secondary metabolites that are gaining

importance for their biotechnological applications. Accordingly, the antimicrobial activity of both fungi was tested. Table (5) showed significance antimicrobial activities of the *Aspergillus niger* EM77 *and Aspergillus awamori* EM66 against *Candida albicans and tropicalis, Pseudomonas* sp. The two isolates had negative effect on *Escherichia coli*. In this concern, it was reported in *Bacillus subtilis* M honey isolate as strong antimicrobial agent against *Candida albicans* ATCC2443 [4]. On contrary, Fawzy et al. [51] reported that the extracellular extracts of

Table 1. Morphological identification of the two isolates

Character	Examination	
	Isolate (A)	**Isolate (E)**
*** Culture exam.: Growth characteristics**	Colonies typically purple black powdery. On dox media reaching 6-7 cm in seven days at 28-30°C.	Colonies on Czapek agar growing rapidly attaining a diameter of 5.0 cm in 7 days, white basal mycelium bearing abundant conidial structures with black color and white at the margin.
*** Microscopic exam. Hyphea**	Hyphae are dark brown and septate, averaging 2.5-4.2 µm in diameter.	
Conidiophores	Conidiophores arising from long, broad, thick walled, branched foot cells, 10-13 µm, in diameters.	Conidiophores thick walled 13.5 µm diameters.
Phialides	Phialides borne on directly on vesicale, born on metulae, conidial head black.	-
Strigmata		Strigmata biseriate, primary (9.0 X 3.0 µm), secondary (7.0 X 2.6 µm).
Vesicles	Vesicles appear globose shape 20-35 µm, diam.,	Vesicle globose, 47 µm in diameter, fertile over their entire surface.
Conidia	Conidia in large, mostly globose to subglobose, black in color, irregularly roughened 4.0-5.0 µm, diam.,uninucleate.	Conidia globose, 4.8 µm.

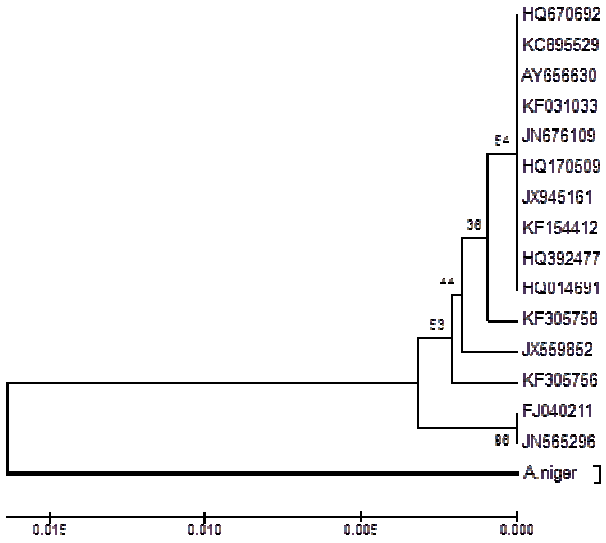

Fig. 1a. Phylogenetic tree for the isolated fungus *Aspragillus niger* and the other *A. niger* isolated fungus listed on gene bank data base. The phylogeny was constructed based on the DNA nucleotide sequence of the 18S rRNA gene. The program was used in the tree construction is Mega4 program

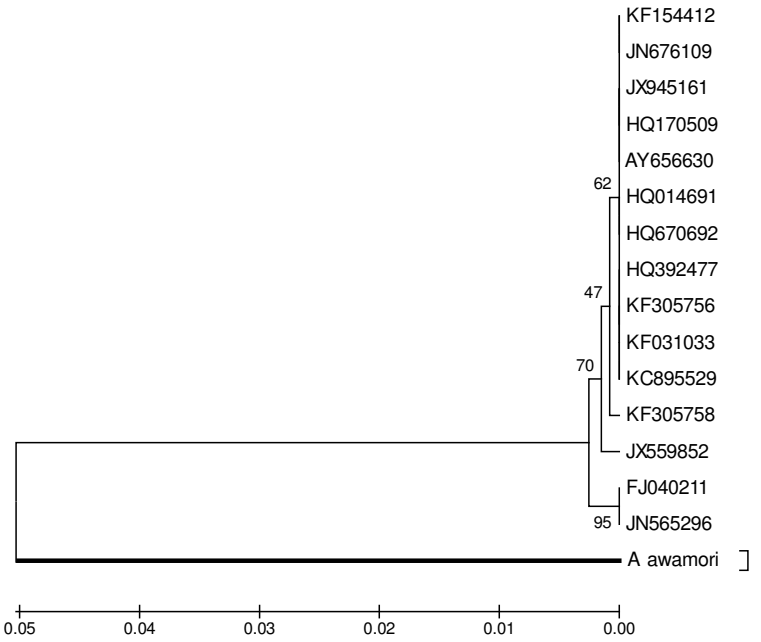

Fig. 1b. Phylogenetic tree for the isolated fungus *Aspragillus awamori* and the other *A. awamori* listed on gene bank database. The phylogeny was constructed based on the DNA nucleotide sequence of the 18S rRNA gene. The program was used in the tree construction is Mega4 program

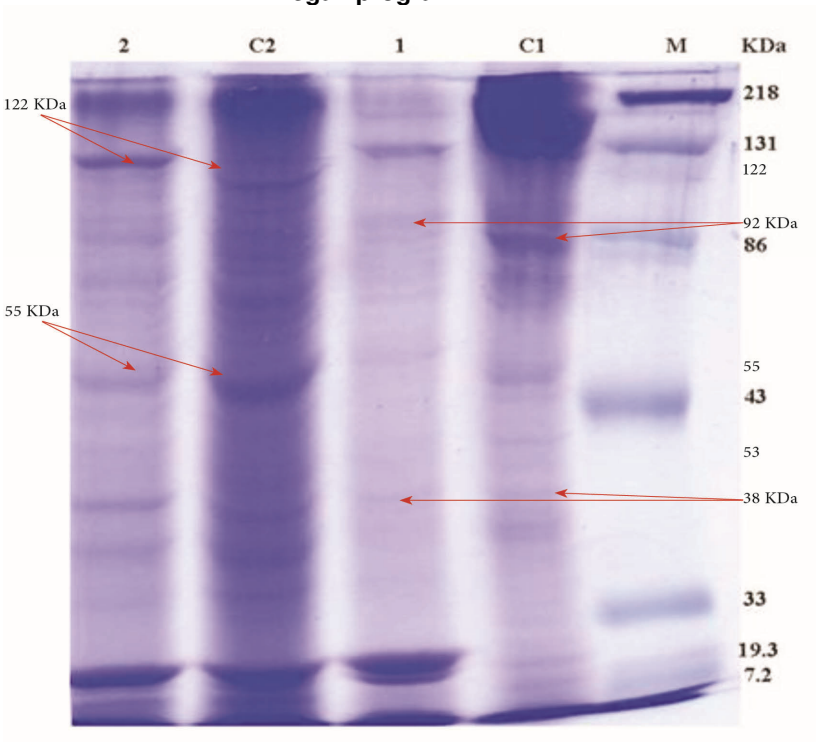

Fig. 2. Electrophoretic protein patterns of the *Aspragillus niger* EM77 (1) and its reference (C1) and *Aspragillus awamori* EM66 (2) and its reference extracts by SDS-PAGE method

Table 2. Number of protein bands under study based on molecular weight (kilodalton) observed by the SDS-PAGE technique: soil isolate *Aspergillus niger* (C1), honey isolate *Aspergillus niger* EM77 (1), soil isolate *Aspergillus awamori* (C2), honey isolate *Aspergillus awamori* EM66 (2)

Band No.	Band molecular weight (kDa)	C1	1	C2	2
1	232	+	−	−	−
2	218	−	+	−	−
3	210	−	−	−	+
4	208	−	−	+	−
5	180	−	+	−	−
6	175	+	−	−	−
7	133	+	−	−	−
8	130	−	+	−	−
9	122	−	−	+	+
10	115	−	−	−	+
11	110	−	−	+	−
12	96	−	−	+	−
13	92	+	+		+
14	87	−	−	+	−
15	85	−	+	−	−
16	84	+	−	−	+
17	81	−	+	−	−
18	77	−	−	+	−
19	76	−	+	−	−
20	74	−	−	+	−
21	72	+	−	−	−
22	71	−	−	−	+
23	67	−	+	−	−
24	65	−	−	+	+
25	60	−	−	+	−
26	59	−	−	−	+
27	55	−	−	+	+
28	52	−	+	−	−
29	47	+	−	−	+
30	45	+	−	+	−
31	41	+	−	−	+
32	39	−	−	+	−
33	38	+	+	−	+
34	37	+	−	+	−
35	36	+	−	−	+
36	35	−	−	+	−
37	34	−	−	−	+
38	33	−	−	+	+
39	19	+	−	−	−
40	17	−	+	−	−
41	8	−	−	+	+
42	7	−	+	−	−
43	5	+	−	−	−
44	2	−	−	+	+

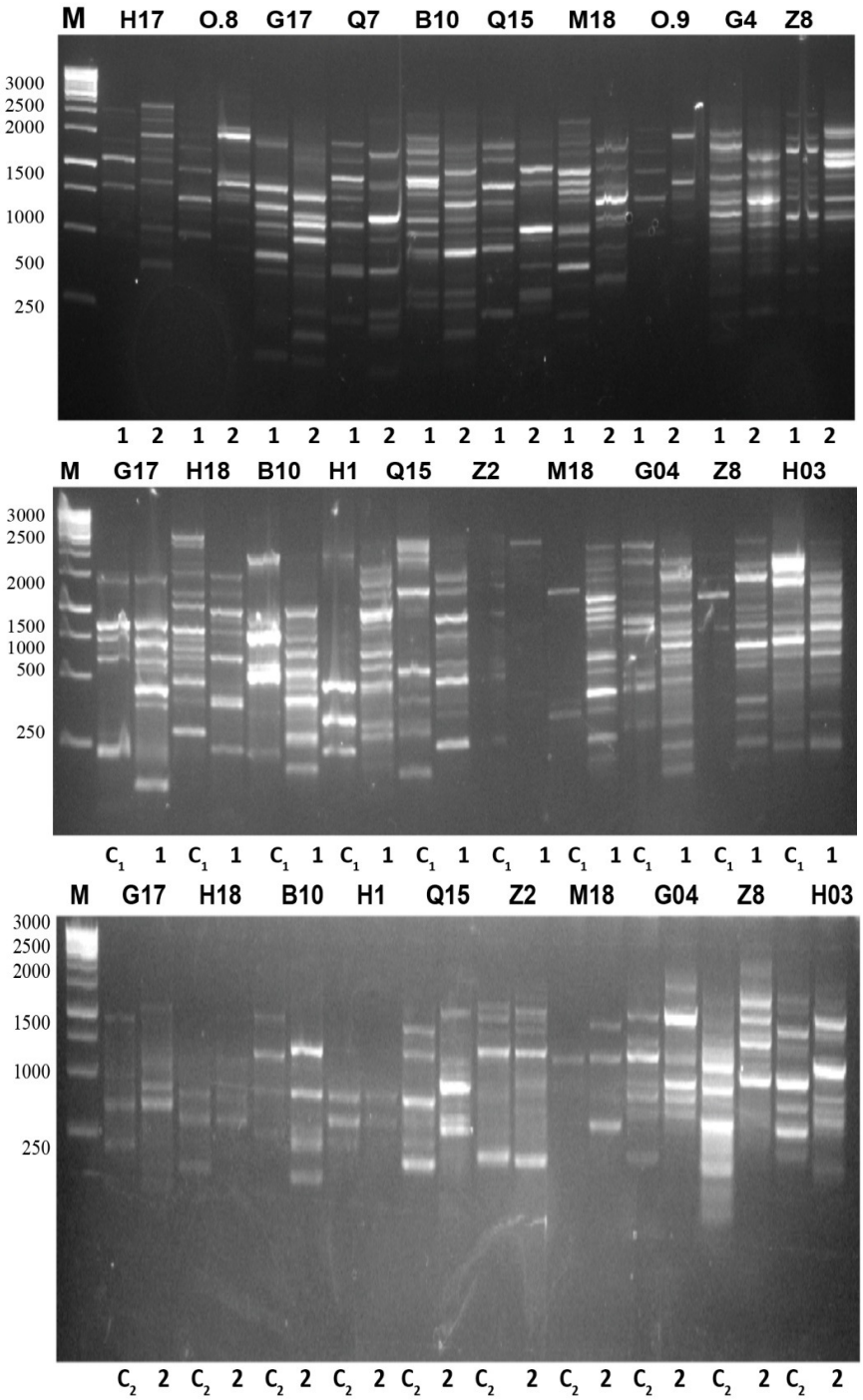

Fig. 3. Amplified polymorphic DNA fragments (bands) that produced by 10 primers in PCR reaction to discriminate the similarity between

a.*Lane 1, Aspergillus niger EM77; and lane 2, Aspergillus awamori EM66.*
b.*Lane C. Aspergillus niger soil isolates and Lane 1 Aspergillus niger EM77 honey isolate.*
c.*Lane C[w]. Aspergillus awamori soil isolates and Lane 2 Aspergillus awamori EM66*

Table 3. Effect of different parameters in honey isolates growth

	Mycelia weight mg			Mycelia weight mg			Mycelia weight mg			Mycelia weight mg			Mycelia weight mg	
pHs	***A. niger***	***A. awamori***	**Temp. (ºC)**	***A. niger***	***A. awamori***	**Carbon sources**	***A. niger***	***A. awamori***	**Nitrogen sources**	***A. niger***	***A. awamori***	**NaCl (%)**	***A. niger***	***A. awamori***
2.0	9.5±0.7	8.5±0.7	30	54.0±1.4	41.5±0.7	without	00	0 0	without	0±0.0	0±0.0	without	59.0±1.4	40±0.7
3.0	19.0±1.4	20.0±2.8	35	46.5±0.7	37.0±1.4	Sucrose	50.5±0.7	42±0.7	$NaNO_3$	42±1.4	41±1.4	2	52.5±3.5	38±1.4
4.0	23.5±2.1	29.0±1.4	40	31.0±1.4	37.0±2.1	Fructose	45.0±1.4	40±1.4	NH_4NO_3	52±1.4	59±1.4	4	44.0±1.4	34±0.7
5.0	40.0±0.7	36.0±0.0	**45**	10.00±	19.5±0.7	Galactose	51.0±1.4	42±1.4	$(NH_4)_3PO_4$	60±0.0	49±2.8	6	40.0±2.8	30±1.4
6.0	42.0±1.4	41.0±1.4	**50**	0	0	Mannose	83.0±4.2	77±1.4	$(NH_4)_2HPO_4$	42±1.4	44±1.4	8	33.0±4.2	26±0.7
6.5	51.0±1.4	.44 ±.0.04	-	0	0	Lactose	44.5±2.1	33±1.4	$NH_4H_2PO_4$	38±2.8	61±1.4	10	22.00.0	22±1.4
7.0	57.5±0.7	46.0±2.8	-	-	-	Glucose	48.5±0.7	41±1.4	NH_4Cl	70±1.4	720.0	12	-	10±0.7
7.5	38.0±2.8	34.0±2.8		-	-	Cellulose	63.0±4.2	47.5±1.6	KNO_3	60±0.7	42±1.4	14	-	-
8.0	31.0±1.4	25.5±0.7				Pectin	69.0±1.4	82±2.8	Yeast extract	55±0.0	43±1.4		-	-
9.0	9.0±1.4	7.5±0.7							Peptone	70±0.0	80±1.4		-	

Table 4. Enzyme activities of the two honey isolates in response to particular substrates

Tested enzymes	**Substrates**	***Aspergillus niger* EM77 U/ml**	***Aspergillus awamori* EM66 U/ml**	**Honey enzyme activity U/ml**
Glucose oxidase	Glucose	1.3±0.04	0	0.4±0.02
Invertase	Sucrose	3.8 ±0.03	3.2±0.02	1.6±0.03
Levansucrase	Sucrose	51 ±0.50	52±0.09	2.0±0.02
Amylase	Starch	3.4±0.04	3.0±0.01	0.3±0.02
Dextranase	Dextran	1.4 ±0.001	0.6±0.003	0.32±0.004
Inulinase	Inulin	2.4±0.004	3.0±0.02	0
Pectinase	Pectin	208±2.20	223±1.40	0
Chitinase	Chitin	25±0.02	44±0.09	0
Protease	Casein	**0**	**0**	0

Table 5. Antimicrobial activity of *Aspergillus awamori* EM66 and *Aspergillus niger* EM77

Isolates	***Aspergillus niger* EM77 (KF774181)**	***Aspergillus awamori* EM66 (KF774180)**
Test organisms	**Zone diameter (mm)**	**Zone diameter (mm)**
Candida tropicalis AINSH	25±0.06	22±0.04
Candida albicans ATCC2443.	15±0.02	12±0.04
Pseudomonas sp.	17±0.03	15±0.01
Escherichia coli ATCC25922	0	0

Aspergillus niger and *Aspergillus flavus* var. showed their activity against Gram-negative bacteria only and they have no effect on *Candida albicans*. Moreover, antioxidant activity was accessed by the reducing power assay. The tow isolates exhibited great antioxidant activities. The results registered 82 & 83% antioxidant activities for *Aspergillus niger* EM77 and *Aspergillus awamor*i EM66, respectively. The antioxidant activity of *Aspergillus* spp. was established by many authors [52].

Often, the capacity for the production of toxic or non-toxic metabolites (e.g., mycotoxins, proteins) needs to be investigated to create a basis for reliable risk assessment [53]. Aflatoxins and ocratoxins test recorded that both the isolates had negative results. It is not surprising to find that none of *Aspergillus* sp. has produced aflatoxins, because the production depends not only of the genetic competence of the strains, but is also influenced by a quite wide range of factors (substrate composition, very low water activity (*Aw*) and acidity of honey) and ecological conditions [54]. Checking for ochratoxin A, a mycotoxin, can reduce any risks and will yield *A. niger* as a safe organism [55].

4. CONCLUSION

This research continues in highlights the honey as a new reservoir of unique isolates. In addition, it was suggested that the honey, with osmophilic stress, carried spores have novel properties. The study referred to the isolation of two fungal spp. that were designed as *Aspergillus niger* EM77 (KF774181) and *Aspergillus awamori* EM66 (KF774180). The molecular study through 18S rRNA sequence analysis, protein pattern and randomly amplified polymorphic DNA PCR patterns (RAPD) was pointed to the isolates novelty. Besides, the biochemical study showed that both isolates were efficient source for important enzymes. Both isolates showed strong antimicrobial and antioxidant activities. The aflatoxins and ocratoxins tests revealed that both isolates had negative results. Accordingly, the biochemical tests confirmed strongly the novelty of these honey isolates, as potential secondary metabolite producers. All the previous results recommended both isolates to be used in industrial application in different aspects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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